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Mutations in the NS5A Gene of Hepatitis C Virus in North American Patients Infected With HCV Genotype 1a or 1b

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Previous studies from Japan have described an association between a conserved sequence within the hepatitis C virus (HCV) genome and resistance to interferon (IFN) therapy for patients infected with HCV genotype 1b [Enomoto et al. (1995): Journal of Clinical Investigation 96: 224–230; Enomoto et al. (1996): New England Journal of Medicine 334:77–81]. The present study examines amino acid sequences surrounding the putative Interferon Sensitivity Determining Region (ISDR) of the NS5A gene of HCV in 21 North American patients with genotype 1a or 1b infection receiving recombinant IFN therapy. The ISDR consensus or intermediate pattern was observed in 13 of 14 NS5A clones from North American patients infected with genotype 1b. However, we found no evidence of the consensus ISDR sequence in any NS5A clones isolated from 15 patients with genotype 1a infection. In select cases, gel shift analysis showed no significant changes in the clonal frequency of the putative ISDR domain of HCV genotype 1a or 1b infected patients who were either nonresponsive to IFN therapy, or relapsed following withdrawal of IFN therapy. These results suggest that a conserved ISDR domain is neither associated with, nor responsible for, IFN resistance in North American patients infected with HCV genotype 1a, and demonstrate a need for further investigation into the reported association between ISDR consensus sequences and IFN resistance in genotype 1b clones. ***J. Med. Virol. 53:118–126, 1997.***

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KEY WORDS: hepatitis C virus; NS5A gene; interferon; interferon sensitivity determining region; gel shift analysis

INTRODUCTION

Worldwide, hepatitis C virus (HCV) is an important cause of chronic hepatitis which can progress to liver cirrhosis and hepatocellular carcinoma. Based on current CDC estimates, approximately 1.9% of the general population in the U.S. have antibodies to HCV [M. Alter, CDC, personal communication]. Primary infection with HCV leads to persistent viremia in approximately 85% of cases and chronic liver disease develops in over 60% of cases. Finally, approximately 20% of individuals with chronic hepatitis C eventually develop medically significant sequelae, including cirrhosis, end-stage liver disease, or hepatocellular carcinoma [Alter, 1995]. Thus, therapeutic management of HCV infection has become an important medical problem.

Recombinant interferon (IFN) is the only FDA approved therapy for chronic hepatitis C in the United States. IFN therapy leads to a clinical remission of hepatitis C in about 40% of cases. Unfortunately, most of the initial responders will relapse when treatment is stopped, so only a small percentage have a long-term sustained response [Davis et al., 1989; Di Bisceglie et al., 1989]. Numerous studies have been conducted to identify host- and viral-related factors important in determining the outcome of IFN therapy. Young age, short duration of disease, absence of cirrhosis, mild activity on biopsy, low gamma-glutamyl transpeptidase (GGTP) levels, and lower body weight were identified as host related predictors of response to IFN [Camps et al., 1993; Davis et al., 1994; Lam et al., 1994; Weiland et al., 1990]. Viral factors also appear to play a substantial role in determining outcome to therapy, at least with respect to current dosing regimens. HCV can be classified into at least six major genotypes and nu-

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merous subtypes, with the predominant genotype in most areas of the world being genotype 1 [Bukh et al., 1995; Simmonds, 1995]. Relevant to the current study, HCV genotype 1b is highly predominant in Japan, while both genotype 1a and 1b are present in the U.S. and Europe. Several studies have found that either a higher pretreatment viral load and/or infection with HCV genotype 1 predicted a poorer response to IFN compared to either a lower pretreatment viral load and/or infection with HCV genotypes 2 or 3 [Craxi et al., 1995; Davis, 1994; Hino et al., 1994; Kanai et al., 1992; Lau et al., 1993; Magrin et al., 1994; Mita et al., 1994; Qu et al., 1994; Takada et al., 1992; Tsubota et al., 1994; Yoshioka et al., 1992]. In multivariate analyses, HCV genotype and high viral load were the only variables that independently predicted the likelihood of treatment failure, stressing the importance of primary virologic factors in determining response to IFN [Chemello et al., 1995; Martinot Peignoux et al., 1995].

HCV has a positive strand RNA genome of ~9.4 kb coding for at least three structural proteins that presumably constitute the viral particle and at least six nonstructural proteins that are presumably expressed in infected hepatocytes [Kolykhalov et al., 1994]. The nonstructural 5 (NS5) gene encodes two proteins, NS5A and NS5B. The NS5B gene product appears to be the viral RNA-dependent RNA polymerase [Behrens et al., 1996]. The function of the NS5A gene product, which is cleaved from the precursor NS5 gene, is not known. Recently, mutations in a region of the NS5A gene of HCV genotype 1b were associated with sensitivity to IFN in Japan [Enomoto et al., 1995]. Pairwise, full length genome comparison of HCV isolated from IFN responsive and nonresponsive patients revealed an accumulation of amino acid differences in the carboxyl-terminal half of the NS5A gene ($NS5A_{2209–2248}$). The region was named interferon sensitivity determining region (ISDR), since a consensus ISDR motif was reportedly predictive of resistance to IFN therapy: Complete response did not occur in any of the patients infected with HCV genotype 1b whose ISDR sequences were identical to that of HCV-J (wild-type), while 13% of patients with one to three changes in the ISDR (intermediate type) had complete responses, and all patients with four to 11 amino acid substitutions (mutant-type) had complete responses [Enomoto et al., 1996]. Preliminary investigations in Europe [Germanidis et al., 1996; Squadrito et al., 1996; Zeuzem et al., 1997] have yielded highly conflicting results relative to the initial reports of Enomoto and colleagues.

The goals of the present study were 1) to study the relationship between NS5A mutations and primary virologic response to interferon therapy in a small subset of North American patients infected with HCV genotype 1a or 1b, who received the standard 6 months course of IFN therapy, and 2) to analyze the ISDR domain before and after IFN therapy using the techniques of nucleotide sequencing or heteroduplex gel shift analysis.

MATERIALS AND METHODS

Patient Population and HCV Testing

We obtained serum samples from 21 patients with active HCV infection. All patients were participating in research studies at the University of Washington Medical Center under written informed consent. Active HCV infection was diagnosed by positive serological testing for HCV antibodies (EIA2, Abbott Laboratories, Abbott Park, IL, and RIBA II, Ortho Diagnostics, Raritan, NJ), and by positive testing for HCV RNA by RT-PCR with primers specific to the 5′ non-coding region and by abnormal biochemical markers as described previously [Gretch et al., 1992, 1993]. Serum was prepared from whole blood within 4 hours of venipuncture and stored at −70°C to allow for optimal recovery of viral RNA. All study patients were treated with IFN for 6 months. The dosage was 3–5 million units of IFN-α 3 times a week.

Virological Classification of Response

Response to IFN therapy was determined by quantitative analysis of HCV RNA levels by the branched DNA (bDNA) assay version 1.0 (Chiron Corp., Emeryville, CA) and by RT-PCR assay before, during, and at the end of IFN treatment as described previously [Gretch et al., 1995]. According to the information given by the manufacturer, 1 HCV Mill. Eq./ml in the bDNA test is equal to the luminescence generated by 10^6 molecules of a 3.2 Kb HCV RNA transcript. Patients were classified as having a complete virologic response to IFN if their serum tested negative for HCV RNA by RT-PCR and by the bDNA assay at the end of IFN therapy. The detection limit of HCV RNA in our RT-PCR assay is less than 100 copies per ml of serum [Gretch et al., 1993]. Response to therapy in one patient with HCV genotype 1a infection (patient 11) was determined by quantitative PCR as described elsewhere [Gretch et al., 1994]. Patients were classified as having a transient virologic response to IFN if their serum tested negative for HCV RNA during therapy, but tested positive for HCV RNA at the end of therapy. Of the six HCV genotype 1b patients, three were nonresponders, one was a responder, and two were transient responders. Of the 15 HCV genotype 1a patients, eight were nonresponders, five were responders, and two were transient responders.

Genotype Analysis

HCV genotyping was performed by RFLP analysis of the 5′-non-coding region as described by Davidson et al. [1995] and by nested PCR analysis of the core gene as described by Okamoto et al. [1992].

RNA Extraction, cDNA Synthesis, and RT-PCR for HCV Genotype 1a and 1b[1]

For analysis of the NS5A gene, total RNA was extracted from patient sera by the single-step gua-

[1]Reaction conditions for HCV genotype 1b are given in brackets { }.

nidinium thiocyanate method [Ausubel et al., 1991] with slight modifications, as described previously [Wilson et al., 1995]. RNA was reverse transcribed in a 25 μl reaction containing 150 pmol of antisense primer 5′-GAGACTTCCGCAGGATTTCT-3′ {5′-CTGGATTTCCGCAGGATCTC-3′}, 3 mM $MgCl_2$, 1 mmol each deoxynucleoside triphosphate (dNTP), 0.6 mM DDT, 75 mM KCL, 50 mM Tris-HCl pH 8.3, 9.4 U RNase inhibitor (Pharmacia LKB, Piscataway, NJ), and 130 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Grand Island, NJ). The mixture was incubated at 37°C for 1 hr, then at 95°C for 5 min. A first round PCR reaction was performed as follows: 10 μl of the cDNA was added to a 40 μl PCR mixture containing 25 pmol of sense primer 5′-TGACGTCCATGCTCACTGAT-3′ {5′-CAGAGACGGCTAAGCGTAGG-3′}, 1.13 mM $MgCl_2$, 23.5 mM Tris-HCl pH 8.3, 35.3 mM KCl and 5 U AmpliTaq polymerase (Perkin Elmer, Norwalk, CT). The reaction was amplified for 30 cycles (94°C for 23 sec, 57.5°C [60°C] for 30 sec, and 72°C for 25 sec) in a Perkin Elmer 9600 thermocycler. A nested PCR was then performed as follows: 1 μl of 1:10 diluted first round PCR product was added to a 49 μl PCR mixture containing 25 pmol of sense primer 5′-CCTCCCATATAACAGCAGAG-3′ {5′-TCCTTGGCCAGCTCTTCAGC-3′}, 25 pmol of antisense primer 5′-CGAAGGAGTCCAGAATCACC-3′ {5′-TCCCTCTCATCCTCCTCCGC-3′}, 3 mM $MgCl_2$, 50 mM Tris-HCl pH 8.3, 75 mM KCl and 5 U AmpliTaq polymerase (Perkin Elmer, Norwalk, CT). The reaction was amplified for 30 cycles (94°C for 23 sec, 55.8°C {60°C} for 30 sec, and 72°C for 25 sec). The amplified product size was confirmed by gel electrophoresis using 2% agarose and molecular weight standards (Gibco BRL, Grand Island, NJ).

Cloning and Sequencing

PCR products were cloned into the TA cloning vector (Invitrogen, San Diego, CA), and plasmids containing ISDR inserts were sequenced as previously described [Gretch et al., 1996; Wilson et al., 1995]. Sequences were analyzed using GCG software (Wisconsin Computer Group). Initially, a single clone was sequenced for patient 1 who was infected with HCV genotype 1b. Additional sequencing of 12 clones from patient 1 indicated that the single clone represented the major ISDR quasispecies variant. For the other HCV genotype 1b patients, two clones were sequenced for patients 3–6. Regarding HCV genotype 1a patients, a single clone, representative of the major ISDR quasispecies variant was sequenced for 12 of 15 patients. For patients 9, 14, and 20, two clones were sequenced from the relapse, post-IFN and post-IFN time points, respectively.

Gel Shift Analysis

Gel shift analysis was performed as previously described [Gretch et al., 1996; Wilson et al., 1995]. The analysis involved mixing, denaturation, and hybridization of a radiolabeled probe with unlabeled target molecules, followed by electrophoresis of the hybrid on nondenaturing polyacrylamide gels. Probe hybridized to itself (unlabeled) served as a marker for identification of homoduplexes. Hybrids with nucleotide changes relative to the probe displayed retarded mobility and were identified as heteroduplexes. To determine the total number of variants in a quasispecies population, the genetic diversity of the individual variants, and their relative abundance, clonal frequency analysis was performed as described [Gretch et al., 1996; Polyak et al., 1997; Wilson et al., 1995]. PCR products from selected time points were ligated into TA cloning vector and individual clones were selected and reamplified to generate clonal PCR products for gel shift analysis. Clonal frequency analysis allows rapid characterization of a quasispecies population. Specifically, this technique permits the screening of a large number of individual clones, allows determination of genetic relatedness, and has the ability to identify minor quasispecies variants.

Classification of ISDR Types in HCV Genotype 1b Clones

According to Enomoto et al. [1996], ISDR amino-acid sequences ($NS5A_{2209–2248}$) derived from HCV genotype 1b can be grouped into three different types: 1) wild type sequences are identical to HCV-J; 2) intermediate type sequences show one to three amino acid changes in the ISDR when compared to HCV-J; and 3) mutant type sequences show four to 11 amino acid changes in the ISDR when compared to HCV-J.

RESULTS

A total of 21 patients with chronic HCV infection were treated with IFN-α for 6 months (3–5 MU/TIW) and HCV RNA levels were monitored quantitatively by bDNA assay or quantitative PCR and qualitatively by RT-PCR (Table I). One of six (16.7%) of the patients infected with HCV genotype 1b (Table I) and five of 15 (33.3%) of patients infected with HCV genotype 1a (Table I) showed a complete virologic response to IFN therapy defined as elimination of detectable viral RNA from serum during and at the end of therapy at a sensitivity of less than 100 RNA copies per ml of serum. Three patients with HCV genotype 1b (50%) and eight patients with genotype 1a infection (53.3%) were found to be nonresponders (Table I). Two patients of the genotype 1b group and two patients of the genotype 1a group were transient responders. These patients showed disappearance of viral RNA by bDNA and/or RT-PCR assay during the course of IFN therapy, but the response was not sustained until the end of therapy. For nonresponsive patients, there appeared to be differences in the rate of change in HCV RNA titers. For example, HCV genotype 1b patients 4 and 6 had significant decreases in HCV RNA titers within the first 3 months of IFN therapy, while patient 5 had HCV RNA titers which remained relatively unchanged. Similarly, HCV genotype 1a patients 14, 15, 19, 20, and

TABLE I. HCV RNA Levels During IFN Therapy in Genotype 1b and 1a Patients*

Patient	Genotype	Response	HCV RNA [Mill.Eq/ml] Before IFN	3 Months of IFN	6 Months of IFN
1	1b	R	26.80	negative	negative
2	1b	TR	3.80	<0.35	<0.35
3	1b	TR	8.20	negative	0.50
4	1b	NR	40.80	5.00	3.90
5	1b	NR	6.20	3.80	2.00
6	1b	NR	44.80	12.70	25.40
7	1a	R	2.90	1.16	negative
8	1a	R	10.90	0.80	negative
9	1a	R	23.10	negative	negative
10	1a	R	4.80	3.20	negative
11[a]	1a	R	50.00	10.00	negative
12	1a	TR	46.80	negative	12.60
13	1a	TR	0.60	<0.35	1.60
14	1a	NR	17.20	1.50	2.50
15	1a	NR	33.20	6.32	2.40
16	1a	NR	3.10	2.80	4.70
17	1a	NR	19.90	20.60	34.00
18	1a	NR	11.80	5.80	22.80
19	1a	NR	47.80	2.30	5.70
20	1a	NR	10.30	0.50	0.60
21	1a	NR	18.70	8.60	1.50

*Changes of the serum HCV RNA concentration during the 6 months of IFN treatment (3–5 MU/TIW) in 21 patients with chronic hepatitis C. Six patients were infected with HCV genotype 1b and 15 patients with HCV genotype 1a. The concentration of HCV RNA in equivalents per mL was determined with the bDNA assay version 1.0 (sensitivity threshold 350,000 Eq./mL) and all bDNA negative specimen were further tested by reverse transcription polymerase chain reaction (RT-PCR). Negative indicates HCV RNA was not detectable by RT-PCR, at a sensitivity threshold of less than 100 copies/ml. A titer of <0.35 was negative in the bDNA assay, but positive by RT-PCR. R, responder; TR, transient responder; NR, nonresponder.
[a]Response to therapy in patient 11 was determined by quantitative PCR.

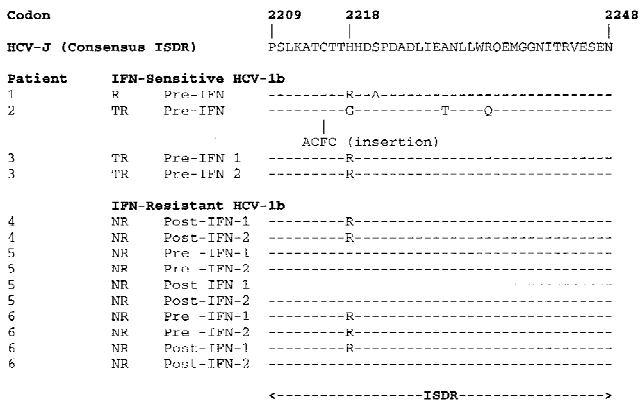

Fig. 1. Deduced amino acid alignment of NS5A for HCV genotype 1b. Deduced amino acid sequence alignment of the NS5A region of the HCV genotype 1b clones for patients 1–6. The sequences are compared to the published sequence of HCV-J, a prototype genotype 1b sequence. Dashes indicate amino acids identical to those found in HCV-J. Codon 2218, a four amino acid insertion (ACFC) in transient responder 1, and the IFN sensitivity determining region (ISDR) described by Enomoto et al. [1995] are indicated. Amino acids are denoted by single letter codes. Sequences are numbered according to HCV-J. R, Responder; TR, transient responder; NR, nonresponder; Pre-IFN, HCV clone obtained before start of IFN therapy; Post-IFN, HCV clone obtained after IFN therapy.

21 had significant decreases in HCV RNA titers within the first 3 months of IFN therapy, while patients 16–18 had HCV RNA titers which remained relatively unchanged. However, because of the persistence of HCV viremia at all time points during IFN therapy these patients were classified as nonresponders.

ISDR in HCV Genotype 1b

The first objective of our study was to test for the presence of the consensus ISDR domain in HCV genotype 1b clones in the U.S. Clones from patients 1–6 (Table I) were selected; NS5A genetic regions were amplified from patients′ sera, cloned, and sequenced as described in Materials and Methods. The deduced amino acid sequences of the HCV genotype 1b clones are aligned in Figure 1 and compared to HCV-J, the prototype 1b clone from Japan which contains the consensus ISDR domain associated with IFN resistance [Enomoto et al., 1995]. All 10 HCV genotype 1b clones we sequenced from the three IFN nonresponders (patients 4–6) showed either the consensus ISDR sequence or a single mutation at codon 2218 (R for H), both of which were associated with IFN resistance in Japan. Clones with amino acid substitutions at codon 2218 fit Enomoto's intermediate type viral category (one to three mutations in the NS5A region). Two clones from patient 3, a transient responder, had a single ISDR mutation (intermediate type) at codon 2218. A single clone from patient 1, a responder, had two mutations in the ISDR (intermediate type) at codon 2218 and 2221. Finally, a single clone from patient 2, a transient responder, had three ISDR mutations plus a 12 nucleotide in-frame insertion at codon 2215 (mutant type). Thus, the ISDR consensus or intermediate pattern was observed in 13 of 14 clones from North American patients infected with genotype 1b.

ISDR in HCV Genotype 1a

The most common HCV genotype in North America is genotype 1a, and data on the ISDR sequence in this genotype has not been published to date. Therefore we analyzed the relationship between amino acid sequence in the ISDR domain of NS5A and response to IFN for 15 patients with genotype 1a infection (patients 7–21, Table I). The deduced amino acid sequences of genotype 1a clones are aligned in Figure 2 and compared to HCV-J, the prototype 1b clone (consensus ISDR). The number of amino acid substitutions in IFN responsive HCV-1a clones when compared to HCV-J ranged from three to five and was thus not different from the range of amino acid substitutions observed in IFN nonresponsive HCV-1a clones (three to five). The clones from transient responders (patients 12 and 13) had three or four amino acid substitutions in the ISDR ($NS5A_{2209–2248}$), while the clones obtained from patients relapsing after IFN therapy (patients 8 and 9) had between four and five amino acid substitutions. We did not detect the consensus ISDR domain in any of the 15 patients with type 1a infection. Further-

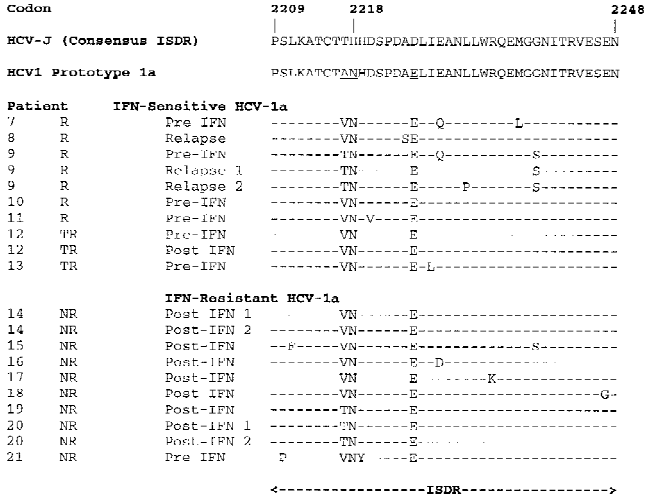

Fig. 2. Deduced amino acid alignment of NS5A for HCV genotype 1a. Deduced amino acid sequence alignment of the NS5A region of the HCV genotype 1a clones for patients 7–21. The sequences are compared to the published sequence of HCV-J, a prototype genotype 1b sequence. Dashes indicate amino acids identical to those found in HCV-J. The sequence of HCV1, a prototype genotype 1a sequence, is also given. The HCV1 prototype 1a sequence differs at the underlined positions from HCV-J (consensus ISDR). Codon 2218 and the ISDR described by Enomoto et al. [1995] for HCV genotype 1b are indicated. Amino acids are denoted by single letter codes. Sequences are numbered according to HCV-J. R, Responder; TR, transient responder; NR, nonresponder; Pre-IFN, HCV clone obtained before start of IFN therapy; Post-IFN, HCV clone obtained after IFN therapy; Relapse, HCV clone obtained at virologic relapse.

more, we failed to detect a specific amino acid substitution pattern or a clustering of mutations around a specific site that would have been characteristic for either IFN responsive or nonresponsive clones.

Comparison of ISDR Before and After IFN Therapy

The sequence of the ISDR in HCV genotype 1b or genotype 1a infection might change during IFN therapy. To address this issue, HCV clones obtained before IFN therapy were compared on a patient by patient basis with clones obtained after IFN therapy or at the time point of virologic relapse for patients 1, 5, and 6 (Figs. 1 and 3), and patients 9 and 12 (Fig. 2). Patient 1, who was infected with HCV genotype 1b, relapsed approximately 2 months after cessation of IFN therapy. To determine if the ISDR sequences which were present at the time point of virological relapse differed from the ones present before IFN therapy, we sequenced three ISDR clones obtained from patient 1 before IFN treatment and nine clones obtained at the time point of relapse (Fig. 3). Before IFN treatment, two different types of ISDR sequences were present. One (Pre-IFN 1) belonged to Enomoto's mutant type group (>4 mutations), the other belonged to the intermediate type (Pre-IFN 2 and 3) [Enomoto et al., 1996]. At the time point of relapse, five different types of ISDR sequences were observed: 78% of the sequences were of intermediate type (relapse 2–8), while 22% of the sequences were of mutant type (relapse 1 and 9). The number of amino acid substitutions in the three pretreatment ISDR sequences from patient 1 (Fig. 3) ranged from two to seven and was thus not different from the range of amino acid substitutions observed in the nine clones obtained at the time point of relapse (2–8). We did not detect a specific amino acid substitution pattern that would have been characteristic for either pretreatment or relapse clones. In particular, no wild type (consensus) ISDR emerged at the relapse time point. Patient 5, a genotype 1b nonresponder, had the consensus ISDR sequence before IFN therapy, and showed no change after therapy (Fig. 1). One of two HCV clones obtained from patient 6 (genotype 1b nonresponder) after IFN therapy showed an amino acid change at codon 2218 (H for R) when compared to the pre-IFN clones. This clone from patient 6 was the only HCV genotype 1b clone that showed an amino acid change towards the consensus ISDR with IFN treatment.

Following cessation of therapy, patient 9 (Fig. 2), who was infected with HCV genotype 1a, suffered a virologic relapse. Analysis of two exemplary clones obtained from patient 9 during relapse showed amino acid sequence differences in the ISDR when compared to the pretreatment clone: one relapse clone differed in one amino acid, the other clone in two amino acids, respectively. The ISDR from patient 12, a genotype 1a transient responder, was identical both before and after IFN therapy.

The clonal frequency analysis technique provides detailed assessment of the level of quasispecies complexity and genetic diversity, because a large number of clones are simultaneously analyzed [Gretch et al., 1996; Polyak et al., 1997; Wilson et al., 1995]. Figure 4 illustrates the changes in HCV quasispecies before and after therapy for patient 9, a HCV-1a responder, and patient 20, a HCV-1a nonresponder. Clonal frequency analysis was used to analyze ISDR sequences. Neither of the patients showed a significant change between

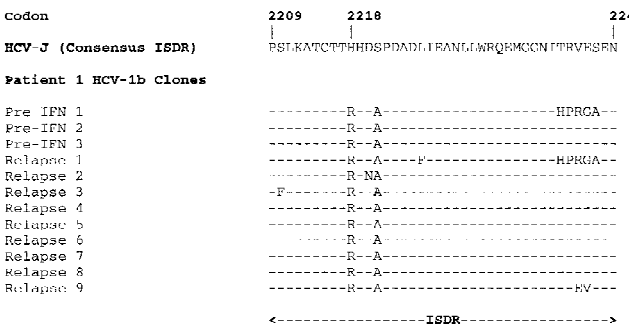

Fig. 3. Analysis of the ISDR for HCV-1b clones obtained from patient 1. Deduced amino acid alignment of the ISDR for HCV-1b clones obtained from patient 1 before IFN treatment and at the time point of relapse. Initially, this patient showed a complete virologic response after 6 months of IFN therapy. The sequences are compared to the published sequence of HCV-J, a prototype genotype 1b sequence. Dashes indicate amino acids identical to those found in HCV-J. Codon 2218 and the ISDR are indicated. Amino acids are denoted by single letter codes. Sequences are numbered according to HCV-J. Pre-IFN, HCV clone obtained before start of IFN therapy; Relapse, HCV clone obtained at virologic relapse.

pre- and posttreatment quasispecies variants. However, a number of distinct gel shift variants were observed at the pretreatment and posttreatment time points for both patients 9 and 20 (pretreatment clones 4, 9, 10, 14 for patient 9; posttreatment clones 4, 7, 14, 15 for patient 20). These experiments indicate the following for patients 9 and 20: 1) There was a quasispecies distribution within the ISDR, and 2) the HCV-1a ISDR quasispecies did not change significantly during IFN therapy.

DISCUSSION

Chronic HCV infection in Japan is most commonly caused by HCV genotype 1b; chronic hepatitis C caused by genotype 1a is rare [Bukh et al., 1995; Takada et al., 1993]. Two recent studies in Japan found a strong positive correlation between mutations in a region of the NS5A gene of HCV genotype 1b ($NS5A_{2209–2248}$) and sensitivity to IFN [Enomoto et al., 1995, 1996]. In contrast to the situation in Japan, HCV genotype 1a is the most common cause of chronic hepatitis C in the United States, although chronic hepatitis C caused by genotype 1b is also common [Bukh et al., 1995; Davidson et al., 1995]. If the preliminary results from Japan were applicable to HCV genotype 1b infection in the U.S., and potentially also to the prevalent genotype 1a infection, the clinical impact in terms of prediction of IFN response would be significant. This is the first study in North American patients with chronic hepatitis caused by HCV genotype 1a or 1b that looks at the correlation between mutations in the NS5A gene and sensitivity to IFN.

Analysis of HCV genotype 1a clones did not reveal any specific amino acid substitution pattern or a clustering of mutations around the ISDR site that would have been characteristic for either IFN responsive or nonresponsive clones. The consensus ISDR sequence defined in Enomoto's studies was not detected in any of the HCV-1a patients. Furthermore, when the NS5A region was analyzed before and after interferon therapy by the heteroduplex gel shift technique, we did not detect any appreciable change in the gel shift pattern in response to interferon pressure. Thus, we found no correlation between the sequence of the ISDR ($NS5A_{2209–2248}$) in HCV genotype 1a and sensitivity to IFN in our analysis of 15 cases.

The main intent of the current study was to investigate ISDR sequences in the more prevalent HCV genotype 1a infections. Thus, our sampling of patients infected with HCV genotype 1b was limited. Based on analysis of only six cases with HCV genotype 1b infection, we found tentative data to support Enomoto's finding of the consensus ISDR sequence being associated with interferon resistance. All genotype 1b IFN responsive or transiently responsive HCV clones showed an amino acid substitution at codon 2218 when compared to the conserved ISDR sequence identified in non-responsive Japanese patients, which correlates with Enomoto's observations that only 13% of patients with one to three changes in the ISDR (intermediate type) had complete responses to IFN, and all patients with 4–11 amino acid substitutions (mutant type) had complete responses [Enomoto et al., 1995, 1996]. Although our study is the first to confirm the presence of the ISDR consensus sequence in IFN resistant genotype 1b infections in North America, we did not study the relationship between ISDR mutations and IFN sensitivity because sustained response to IFN was not observed in our cohort.

In this study, we used cloning and sequencing of the ISDR to examine the relationship between ISDR mutations and response to IFN therapy. In contrast, Enomoto and colleagues [1995, 1996] used direct sequencing for their studies. We have also analyzed the ISDR in additional HCV genotype 1a patients by direct sequencing and obtained results similar to cloning and sequencing (data not shown); therefore our results are most likely comparable to that of Enomoto and colleagues.

We have recently shown that the NS5A protein of HCV interacts with and inhibits the activity of the IFN-induced RNA activated Protein Kinase, PKR, a key mediator of the IFN induced antiviral state [Gale et al., 1997]. The interaction was ISDR-dependent and PKR bound to NS5A from both HCV-1b and HCV-1a clones. The HCV-1a NS5A clone differed from the HCV-1b NS5A clone by three amino acids in the ISDR. These results are consistent with the clinical observations that the consensus ISDR motif is associated with interferon resistance in Japan, and with our preliminary data on HCV-1b ISDR in North America. Therefore, NS5A may play at least a partial role in the generalized resistance of HCV genotype 1 to standard doses of IFN. However, we did not find a clear association between specific ISDR mutation patterns and response to IFN therapy in HCV-1a infected patients: all of the HCV-1a ISDR sequences differed from the HCV-1b ISDR by at least three amino acids (average = 3.8 amino acid changes). It is possible that elevated doses of IFN are required in order to demonstrate the existence of the ISDR in HCV-1a infected individuals. Alternatively, it is possible that NS5A mutations may influence response to interferon in a different manner for HCV genotype 1a compared to 1b.

Nucleotide sequencing and heteroduplex gel shift analysis revealed that the ISDR in HCV genotype 1a and 1b infected individuals exists as a quasispecies (i.e., multiple genetic variants within an individual patient). One prediction from the results of Enomoto and colleagues would be that a viral quasispecies population which contained a mixture of resistant and sensitive ISDR variants, when subjected to IFN pressure, would be enriched for IFN-resistant ISDR variants. This did not appear to occur for patient 1 (Fig. 3) who was infected with HCV-1b. Selection for IFN-resistant ISDR sequences was also not observed for HCV-1a patients (Figs. 2 and 4). Recent reports from Japan indicate some patients with no mutation in the ISDR still cleared viremia after IFN therapy [Chayama et al., 1997]. Studies in Europe also could not confirm the

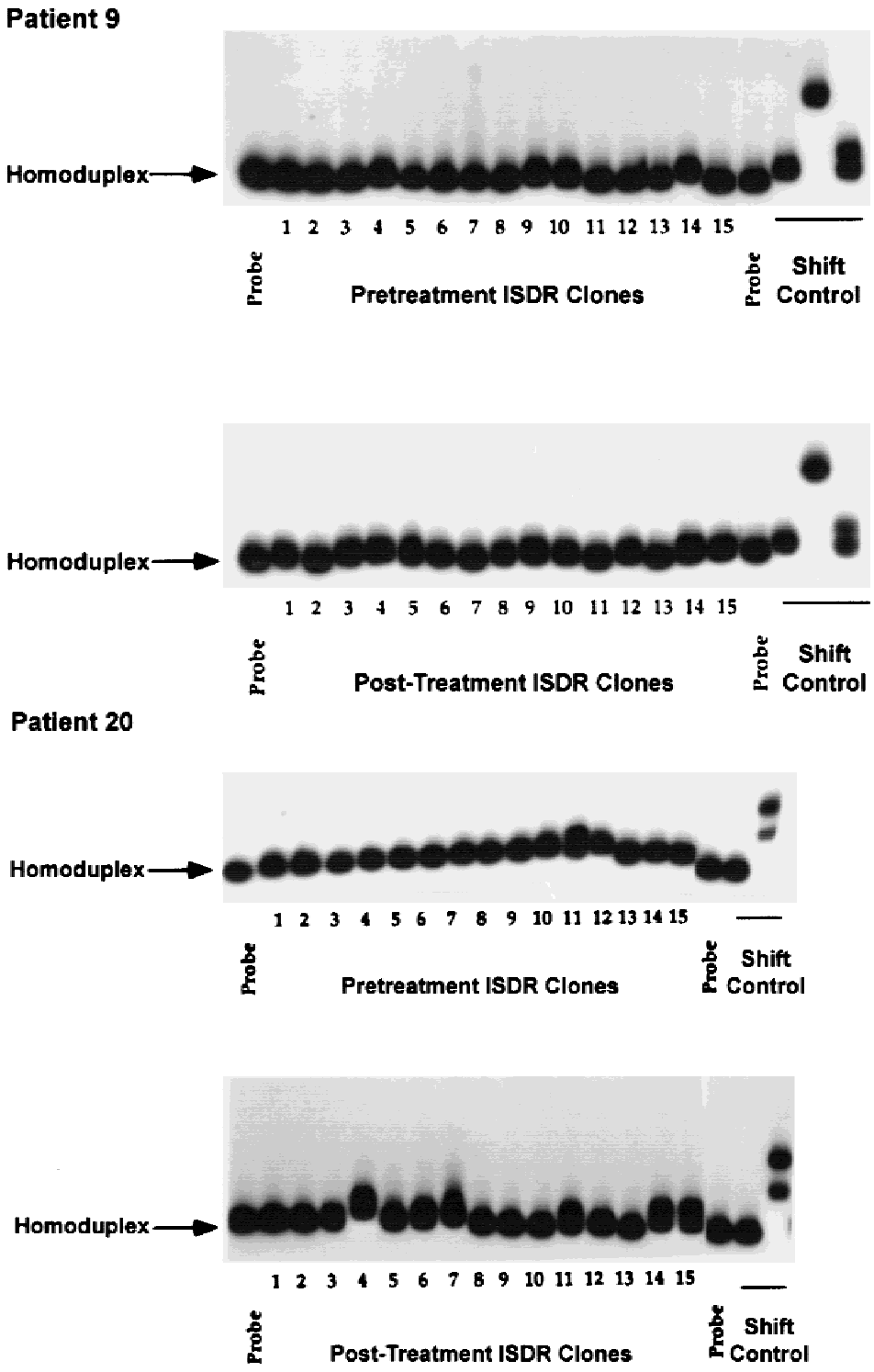

Fig. 4. Clonal frequency analyses for patients infected with HCV-1a. Clonal frequency analysis of the ISDR before and after IFN therapy for patient 9, a HCV-1a responder, and patient 20, a HCV-1a non-responder. Radiolabelled ISDR probes corresponding to pretreatment quasi-species major variants were hybridized to ISDR PCR products derived from individual recombinant ISDR molecules, and gel shift analysis was performed as described in Materials and Methods. The homoduplex control is indicated by an arrow. Individual clones are numbered below each lane.

presence of consensus ISDR sequences in IFN-resistant genotype 1a or 1b infections [Zeuzem et al., 1997], and similar results have also been reported from other European countries in abstract form [Germanidis et al., 1996; Squadrito et al., 1996]. Thus, as reviewed by Herion and Hoofnagle [Herion and Hoofnagle, 1997], it is possible that the ISDR represents only one component of viral and host factors which contributes

to IFN sensitivity. Clearly, additional HCV-1b clones need to be analyzed in detail, perhaps by the quasispecies clonal frequency analysis technique [Gretch et al., 1996; Polyak et al., 1997; Wilson et al., 1995]. Until more extensive data is gathered using reliable techniques, the question of NS5A sequences associated with IFN resistance in hepatitis C remains controversial.

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